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Synthesis, characterization and DNA-binding properties of Ru(II) complexes coordinated by ofloxacin as potential antitumor agents

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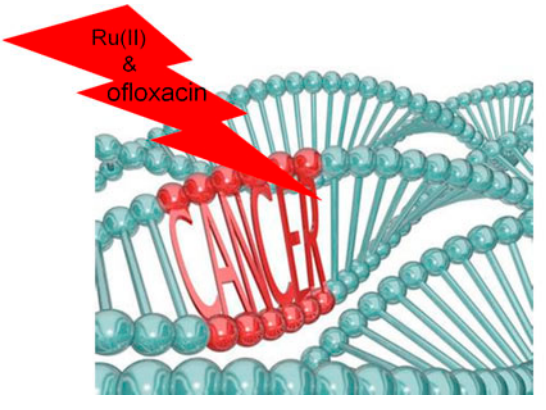

Three Ru complexes coordinated by oxfloxacin, $[Ru(L)_2(OFX)]Cl{\cdot}2H_2O$ (L = bpy, **1**; dmbpy, **2**; phen, **3**; and OFX = ofloxacin), were synthesized and characterized. These complexes can inhibit the growth of cervical cancer HeLa cells efficiently. Furthermore, these three complexes exhibited excellent binding affinities with DNA, as confirmed by spectroscopy methods and viscosity experiments. Therefore, the synthesized Ru(II) complexes have excellent DNA-binding abilities with potential applications in cancer chemotherapy.

Keywords: Ru(II) complexes; Ofloxacin; Antitumor; DNA-binding

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1. Introduction

Quinolones, a commonly used term for quinolone carboxylic acids or 4-quinolones, are a group of antibiotics that inhibit the action of DNA topoisomerase, which catalyzes the conversion of supercoiled DNA into a negatively supercoiled form [1, 2]. Ofloxacin is one of the most frequently used 4-quinolone synthetic antibiotics and exhibits a good distribution pattern in low dose levels [3]. Ofloxacin is used in the treatment of several infectious diseases, such as typhoid fever, meningococcal infection, and recently, multibacillary leprosy [4–6]. The use of ofloxacin for urinary tract infection treatment has increased in the past decade because this antibiotic has excellent efficacy and increases resistance to older agents, such as trimethoprim and sulfamethoxazole [7]. Some studies have reported that ofloxacin could efficiently inhibit the growth of various tumor cells via induced apoptosis [8, 9].

Considerable evidence suggests that ruthenium(II)–polypyridyl complexes can kill tumor cells by interacting with DNA and inducing apoptosis [10–12]. Ji and co-workers showed that Ru(II)–polypyridyl complexes bearing 1H-imidazo[4,5-*f*][1, 10]-phenanthroline analogs, such as $[Ru(bpy)_2(bfipH)]^{2+}$ and $[Ru(phen)_2(bfipH)]^{2+}$ (bpy = 2,2′-bipyridine; phen = 1,10-phenanthroline; and bfipH = 2-(benzofuran-2-yl)imidazo[4,5-*f*][1, 10]phenanthroline), showed high binding affinities toward DNA, attributed to the planarity of the intercalating ligand [13]. Studies by our research group also found that Ru(II)–polypyridyl complexes with tFMPIP (=2-(trifluoromethylphenyl)-1H-imidazo[4,5*f*][1, 10]-phenanthroline) can regulate the expression of the Bcl-2 family protein to induce apoptosis of tumor cells through the caspase signal pathway [14]. Whether Ru(II)–polypyridyl complexes with ofloxacin can also exhibit antitumor activity would be a significant finding [15].

In this study, three Ru(II)–ofloxacin complexes (scheme 1) were synthesized and characterized. The antitumor activity and DNA-binding properties of these Ru(II) complexes were investigated to evaluate their potential application in chemotherapy. The results indicated that this class of complexes could effectively inhibit the growth of tumor cells. Spectroscopic and viscosity studies further indicated that these complexes bind to CT DNA through a classical electrostatic effect. The inhibitory effect of the complexes against HeLa cells agrees with the DNA-binding ability, wherein the increase in substituent group or aromatic ring can enhance the antitumor activity and DNA affinity.

2. Experimental

2.1. *Chemicals*

Ru(III) chloride hydrate was obtained from Mitsuwa Chemicals. 2,2′-Bipyridine, 4,4′-dimethyl-2,2′-bipyridine and 1,10-phenanthroline were purchased from Aldrich, while ofloxacin was purchased from Vega Pharma Co., Ltd. All chemicals, including solvents, were obtained from commercial vendors and used as received. Calf thymus DNA (CT DNA) was purchased from Guangzhou Ruizhen Biotechnology Co. All aqueous solutions were prepared with double distilled water. The Tris–HCl buffer was made from tris (hydroxymethyl)aminomethane, Tris (617 mg), and NaCl (292.5 mg), and was adjusted to pH 7.2 using HCl (0.1 M); this buffer was used for ultraviolet and fluorescence spectroscopic titration and viscosity measurements. The CT DNA purity was checked by monitoring the absorption ratio at 260/280 nm (A_{260}/A_{280}). The ratio was 1.88, indicating that CT DNA was sufficiently free from protein.

2.2. *Instruments*

Electrospray ionization mass spectra (ESI-MS) were recorded on a LCQ DECA XP system (Thremo, USA). ^{1}H NMR spectra were recorded on a Varian-300 spectrometer. All chemical shifts were relative to tetramethylsilane. Ultraviolet (UV) titration was recorded on a Shimadzu UV-2550 spectrophotometer; the steady-state emission spectra were recorded on a RF-5301 fluorescence spectrophotometer.

2.3. *Synthesis of complexes*

2.3.1. Synthesis of *cis*-[Ru(bpy)$_2$Cl$_2$]·2H$_2$O. *Cis*-[Ru(bpy)$_2$Cl$_2$]·2H$_2$O was prepared according to the literature procedure [16]. Solution containing 2,2′-bipyridine (312 mg, 2 mM), RuCl$_3$·3H$_2$O (261.5 mg, 1 mM), and LiCl (127 mg, 3 mM) in a solvent mixture of ethylene glycol and distilled water (V : V = 9 : 1, 30 mL) was heated at 140 °C under Ar for 4 h. Acetone (50 mL) was then added to the solution and left at 4 °C for the whole night. The product was obtained after filtration and drying.

2.3.2. Synthesis of *cis*-[Ru(dmbpy)$_2$Cl$_2$]·2H$_2$O. *Cis*-[Ru(dmbpy)$_2$Cl$_2$]·2H$_2$O was prepared following the same method, but 4,4′-dimethyl-2,2′-bipyridine (368 mg, 2 mM) replaced 2,2′-bipyridine (312 mg, 2 mM).

2.3.3. Synthesis of *cis*-[Ru(phen)$_2$Cl$_2$]·2H$_2$O. *Cis*-[Ru(phen)$_2$Cl$_2$]·2H$_2$O was prepared following the same method, but 1,10-phenanthroline (356 mg, 2 mM) replaced 2,2′-bipyridine (312 mg, 2 mM).

2.3.4. Synthesis of [Ru(bpy)$_2$(OFX)]Cl·2H$_2$O (1). [Ru(bpy)$_2$(OFX)]Cl·2H$_2$O was synthesized according to the literature procedure with some modifications [17]. *Cis*-[Ru(bpy)$_2$Cl$_2$]·2H$_2$O (104 mg, 0.2 mM), ofloxacin (100 mg, 0.3 mM), and CH$_3$CH$_2$ONa (34 mg, 0.5 mM) were added into an ethanol solution (50 mL) and refluxed under Ar for 3 h. The pH of the solution was adjusted to 7 using dilute hydrochloric acid and the product was dried by rotary evaporation. The brown-red crude product was then obtained by adding CH$_3$CN, collecting the precipitate, and drying in a vacuum. The crude product was dissolved in CH$_3$CN and purified in a silica gel column using CH$_3$CN : CH$_3$CH$_2$OH (V : V, 10 : 1) as an eluant. The second brown-red band was collected and dried in a vacuum in 70% yield. The following were found when calculations were conducted for C$_{38}$H$_{35}$ClFN$_7$O$_4$Ru·2H$_2$O: C 54.00%, H 4.65%, and N 11.60%. Found: C 53.96%, H 4.71%, and N 11.54%. ^{1}H NMR (ppm, D$_2$O, 400 MHz) δ: 8.84 (d, 1H, J = 3.2), 8.37–8.32 (m, 4H), 8.07 (t, 2H, J = 8.4), 7.81–7.75 (m, 4H), 7.61–7.58 (m, 2H), 7.52 (t, 2H, J = 9.6), 7.12–7.10 (m, 1H), 4.46–4.41 (m, H), 4.26–4.17 (m, 2H), 3.13–3.04 (m, 4H), 2.48–2.31 (m, 4H), 2.17(s, 1H), and 1.40 (d, 3H, J = 6.8).

2.3.5. Synthesis of [Ru(dmbpy)$_2$(OFX)]Cl·2H$_2$O (2). [Ru(dmbpy)$_2$(OFX)]Cl·2H$_2$O was prepared following the same methods, but *cis*-[Ru(dmbpy)$_2$Cl$_2$]·2H$_2$O (115 mg, 0.2 mM) and ofloxacin (100 mg, 0.3 mM) were used and 69% yield was obtained [18]. Calculated

for $C_{42}H_{43}ClFN_7O_4Ru{\cdot}2H_2O$: C 60.80%, H 5.22%, and N 11.80%. Found: C 60.30%, H 5.32%, and N 11.40%. ^{1}H NMR (ppm, D_2O, 400 MHz) δ: 8.85 (d, 1H, $J = 3.2$), 8.26 (d, 2H, $J = 12$), 8.15–8.10 (m, 2H), 7.49–7.46 (m, 2H), 7.45–7.43(m, 2H), 7.31 (d, 2H $J = 4.4$), 7.26 (d, 2H, $J = 4.8$), 7.06–7.03 (m, 1H), 4.51–4.47 (m, H), 4.31–4.20 (m, 2H), 3.11–3.07 (m, 4H), 2.49–2.46 (m, 4H), 2.41 (s, 3H), 2.39–2.34 (m, 6H), 2.21–2.13 (m, 6H), and 1.46 (d, 3H $J = 5.6$).

2.3.6. Synthesis of [Ru(phen)$_2$(OFX)]Cl·2H$_2$O (3). [Ru(phen)$_2$(OFX)]Cl·2H$_2$O was prepared following the same methods, but *cis*-[Ru(phen)$_2$Cl$_2$]·2H$_2$O (113 mg, 0.2 mM) and ofloxacin (100 mg, 0.3 mM) were used and 64% yield was obtained (calculated according to Ru). Calculated for $C_{42}H_{35}ClFN_7O_4Ru{\cdot}2H_2O$: C 56.50%, H 4.40%, and N 10.98%. Found: C 56.30%, H 4.48%, and N 10.91%. ^{1}H NMR (ppm, D_2O, 400 MHz) δ: 8.87(s, 1H), 8.58–8.53 (m, 4H), 8.27(d, 2H, $J = 8.8$), 8.23(d, 2H, $J = 6.8$), 8.10–8.00 (m, 4H), 7.74–7.68 (m, 4H), 7.22–7.19 (m, 1H), 4.45–4.40 (m, H), 4.24–4.11 (m, 2H), 2.89–2.64 (m, 4H), 2.24–2.13 (m, 4H), 2.06 (s, 3H), and 1.45 (d, 3H $J = 5.6$).

2.4. *MTT assay*

The compounds were evaluated *in vitro* with a 3-(4,5-dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide (MTT) cell viability assay with cervical cancer HeLa cells. All complexes were dissolved in DMSO with stock solution at 20 mM. Cell viability was determined by measuring the ability of cells to transform MTT into a purple formazan dye [19]. Cells were seeded in 96-well tissue culture plates for 24 h. The cells were then incubated with the tested compounds at different concentrations (400, 200, 100, 50, 25, 12.5, and 6.25 μM, with concentration of DMSO of 2% in control and experiment group) for different periods. After incubation, 20 mL of MTT solution (5 mg mL^{-1}) in phosphate buffered saline, PBS was added to each well, and then incubated for 5 h. The medium was aspirated and replaced with a 150 μL/well of DMSO to dissolve the formazan salt formed. The color intensity of the formazan solution, which reflects the cell growth condition, was measured at 570 nm using a microplate spectrophotometer (SpectroAmax TM 250).

2.5. *DNA-binding properties*

2.5.1. Electronic spectra. Electronic absorption spectral experiments were conducted at room temperature to determine the binding affinity between DNA and the compounds. The titration processes were repeated until the spectra did not change for at least four titrations, indicating that binding saturation was achieved. The intrinsic binding constants [20] for the complexes with CT DNA at intraligand (IL) band were calculated following equation (1) [21] and according to the decay of IL band in the presence of DNA [19],

$$(\varepsilon_a - \varepsilon_f)/(\varepsilon_b - \varepsilon_f) = \left[b - (b^2 - 2K^2C_t[\mathrm{DNA}]/S)\right]^{1/2}/2KC_t \quad (1)$$

$$b = 1 + KC_t + K[\mathrm{DNA}]/2S \quad (2)$$

where [DNA] is the concentration of DNA in the base pair, ε_a is the extinction coefficient observed for the absorption band at the given DNA concentration, ε_f is the extinction coefficient of the complex free in the solution, and ε_b is the extinction coefficient of the complex when fully bound to DNA. A plot of [DNA]/[$\varepsilon_a - \varepsilon_f$] *versus* [DNA] produced a slope 1/[$\varepsilon_a - \varepsilon_f$] and a Y intercept equal to 1/(K_b[$\varepsilon_b - \varepsilon_f$]), respectively. The intrinsic binding constant K_b is the ratio of the slope to the intercept.

2.5.2. Fluorescence emission titrations. Fluorescence experiments were conducted by adding small aliquots of DNA solution to Ru(II) complexes. Samples were excited at 340 nm and emission was observed between 500 and 700 nm. After the solutions were mixed for 2 min, absorption spectra were recorded [22].

2.5.3. Viscosity measurements. The viscosity measurement is an effective method to judge the interaction mode of complexes with DNA. Fixed solutions of complexes and DNA in different concentrations were prepared in Tris–HCl buffer media, with [complex]/[DNA] = 0, 0.02, 0.04, 0.06, 0.08, 0.1. Before testing, the solutions were stored in a thermostatic water bath at (30 ± 0.1 °C) 1 h. The formula calculated viscosity is: $\eta = (t - t_0)/t_0$. Viscosity curves were obtained using $(\eta/\eta_0)^{1/3}$ as the Y-axis and with r (r = [complex]/[DNA]) as the X-axis [23].

3. Results and discussion

3.1. *Synthesis and characterization*

The objective compounds were prepared with a yield of approximately 70%. The ESI-MS exhibited for **1** a peak at *m/e* 774.2 (100%) and 387.7 (75%) ascribed to $[M\text{-}2Cl^{-}\text{-}H^{+}]^{+}$ and $[M\text{-}2Cl^{-}]^{2+}$; for **2**: a peak at *m/e* 830.33 (100%) and 415.73 (70%) ascribed to $[M\text{-}2Cl^{-}\text{-}H^{+}]^{+}$ and $[M\text{-}2Cl^{-}]^{2+}$; and for **3**: a peak at *m/e* 822.27 (100%) and 411.73 (85%) ascribed

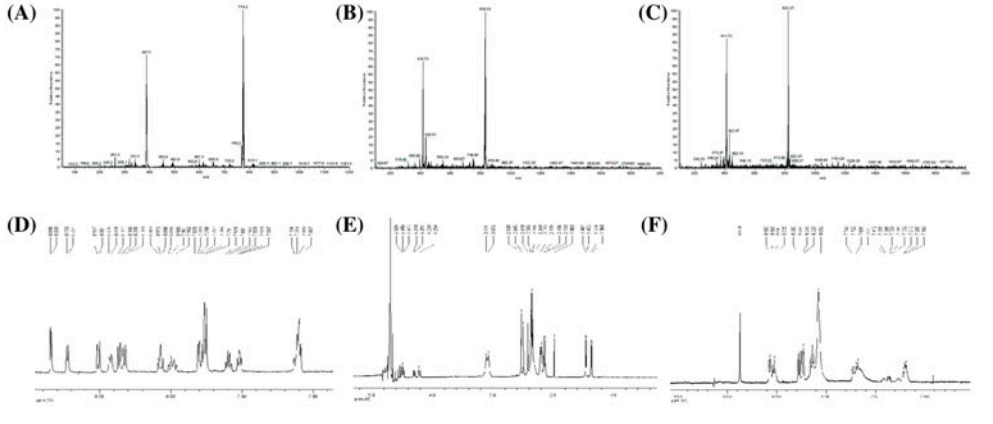


Figure 1. The ESI-MS spectra of (A) **1**, (B) **2**, and (C) **3**; the ^{1}H NMR spectra of (D) **1**, (E) **2**, and (F) **3**.

to $[M\text{-}2Cl^{-}\text{-}H^{+}]^{+}$ and $[M\text{-}2Cl^{-}]^{2+}$, respectively, which agreed with the theoretical values [figure 1(A)–(C)]. These results may be attributed to the structural differences between three complexes which have different bipyridine or phenanthroline coordinated to Ru. The ^{1}H NMR spectrum of ofloxacin exhibited the same signals in the three complexes, which were assigned with typical peaks between 2 and 5 ppm [24], in agreement with the similar structure of arene ruthenium complexes with quinolones [25]. For **1–3**, the typical signals of 2,2′-bipyridine, 4,4′-dimethyl-2,2′-bipyridine, and 1,10-phenanthroline were assigned between 7 and 9 ppm with some differences, which is in agreement with similar arene ruthenium complexes with quinolones [25]. The clear characterization of the complexes' structures facilitates the understanding of their anticancer mechanisms [26].

3.2. *Studies on the antitumor activities*

The antitumor activity of **1–3** against human cervical cancer HeLa cells was evaluated through MTT assay. The inhibitory effects of the synthesized Ru(II) complexes and the ligand ofloxacin on HeLa cell after a treatment of 72 h were demonstrated. As shown in figure 2, **1**, **2**, and **3** displayed an acceptable antiproliferative effect against HeLa cells, which was better than that of ofloxacin. Complexes **1–3** exhibited higher inhibitory effects than ofloxacin at the same concentrations of 50 and 100 μM. Moreover, **3** exhibited the best inhibition, which indicated that increasing of the aromatic ring can enhance the antitumor activity of Ru(II) complexes [27–29].

3.3. *DNA-binding properties*

DNA is considered as one potential target of Ru(II) complexes that inhibit the growth of tumor cells. Investigation on the interaction of these complexes with CT DNA may help explain the potential antitumor mechanism. In addition, the DNA-binding properties of these Ru(II) complexes were evaluated using electronic and emission spectra, and viscosity measurements.

3.3.1. Electronic spectra. The electronic spectra of ofloxacin, **1**, **2**, and **3** in the absence and presence of CT DNA are illustrated in figure 3. As shown in figure 3, the characteristic band at 287 nm in the electronic spectra of ofloxacin was attributed to the $\pi \rightarrow \pi^{*}$ electron

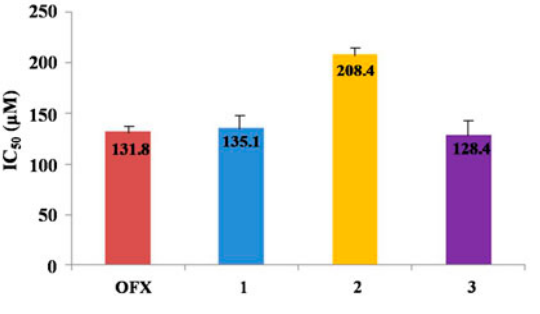


Figure 2. The inhibitory effect of ofloxacin (OFX), **1**, **2**, and **3** on HeLa cells.

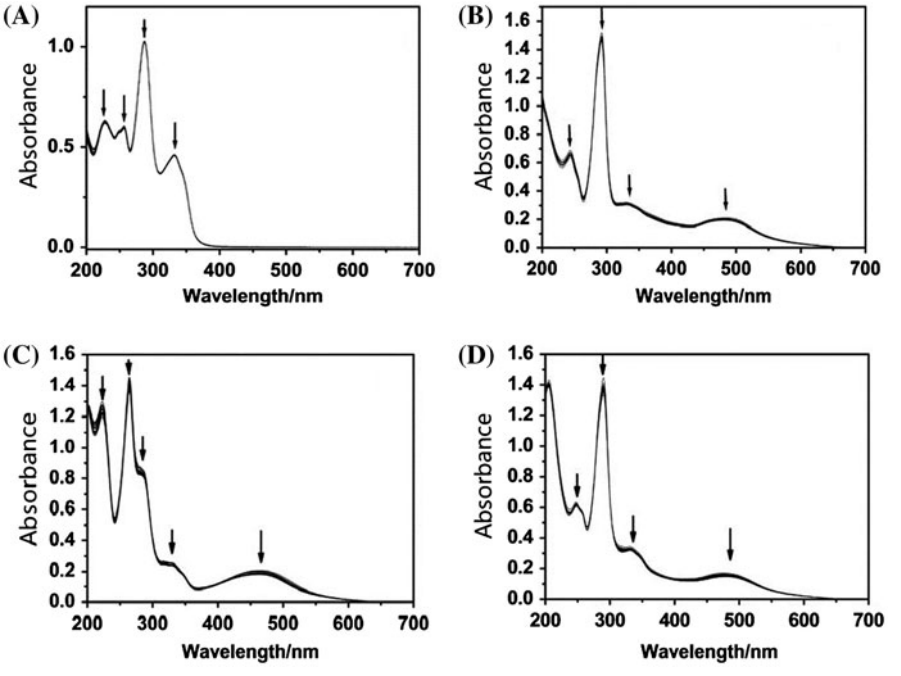


Figure 3. Electronic absorption spectra of (A) ofloxacin, (B) **1**, (C) **2**, and (D) **3** in Tris–HCl buffer (pH 7.2) in the absence and presence of CT DNA. [Compound] = 20 μM, [DNA] = 1 mM. The arrows show the absorption intensity changes upon increasing concentration of CT DNA.

transfer and three other shoulder bands were observed at 332, 257, and 227 nm. For **1**, a moderate band was observed at 480 nm, which was attributed to metal-to-ligand charge transfer (MLCT). The IL $\pi \rightarrow \pi^*$ electron transfer shifted to 292 nm and the shoulder absorption band at 332 nm decreased. A similar change occurred in **2** and **3**, and the MLCT band appeared at 475 and 464 nm, respectively, indicating that the electron circumstance changed when ofloxacin coordinated to the Ru(II).

As shown in figure 3, the electronic absorption spectra of ofloxacin were almost unchanged when CT DNA was added. However, hypochromism and red-shift occurred in the spectra of the complexes. The hypochromism for **1**, **2**, and **3** is 8% ($\Delta\lambda = 4$ nm), 12% ($\Delta\lambda = 5$ nm), and 14% ($\Delta\lambda = 4$ nm) and the binding constants (K_b) for **1**, **2**, and **3** are 1.12×10^4, 1.78×10^4, and 2.36×10^4 M^{-1}, respectively. These results indicate that the complexes exhibit certain affinities to duplex CT DNA [10, 27, 30, 31]. Importantly, **3** exhibited the strongest affinity, which agrees with the antitumor activity.

3.3.2. Fluorescence emission titrations. The DNA-binding properties of these Ru(II) complexes have also been investigated using steady-state emission fluorescence spectra, and the results are shown in figure 4. When excited at 284 nm, all Ru(II) complexes, including ofloxacin, exhibited emission spectra from 350 to 650 nm. The emission maxima for ofloxacin and **1**, **2**, and **3** are at 452, 449, 452, and 451 nm, respectively. The emission intensity of ofloxacin gradually decreased when CT DNA was added. Upon addition of CT DNA, the emission intensity in spectra of the complexes increased. These results may be attributed

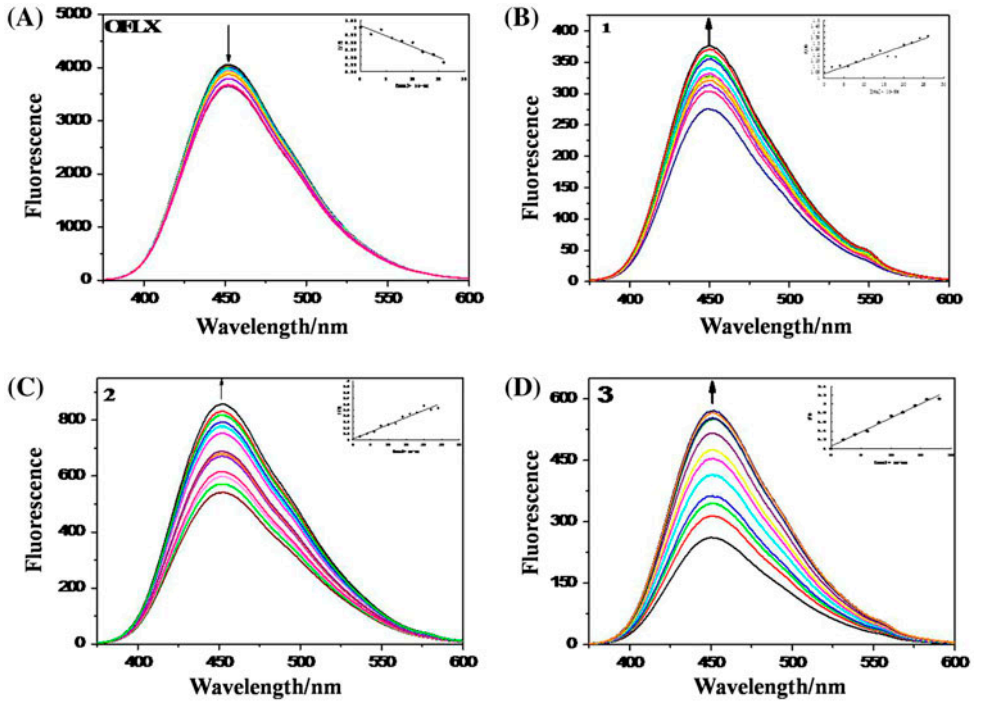


Figure 4. Emission spectra of (A) ofloxacin, (B) **1**, (C) **2**, and (D) **3** in Tris–HCl buffer (pH 7.2) in the absence and presence of CT DNA. [Compound] = 20 μM, [DNA] = 1 mM. The arrows show the emission intensity changes upon increasing concentration of CT DNA.

to the fact that the Ru(II) moiety was protected by the hydrophobic DNA molecule to be quenched by water, and the relative emission intensity (I/I_0) for ofloxacin, **1**, **2**, and **3** at the ratio of [DNA]/[Ru] = 1.0 are 0.89, 1.36, 1.58, and 2.18, respectively. These results indicate

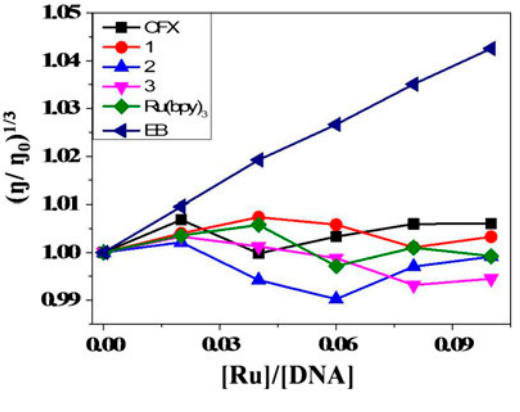
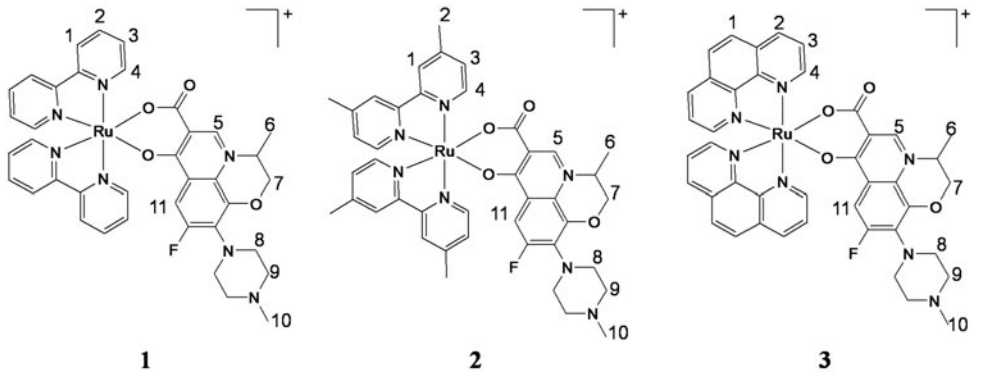


Figure 5. Effect of (■) ofloxacin, (●) **1**, (▲) **2**, (▼) **3**, (♦) $[Ru(bpy)_3]^{2+}$, and (◄) EB on the relative viscosity of CT DNA.



Scheme 1.

that the binding affinity increased in the sequence ofloxacin < **1** < **2** < **3**, which is attributed to the hydrophobic and steric hindrance of the co-ligand [32]. These data also agree with the aforementioned study, indicating that the DNA-binding behavior is related to the antitumor activity of the Ru(II) complexes [33].

3.3.3. Viscosity measurements. The relative viscosity of DNA increases with addition of metal complexes that bind through intercalation. Viscosity measurements have proven to be an important method to study the mode of binding to DNA. In general, the intercalation of a compound into DNA causes an observable increase in the viscosity of a DNA solution because of the increase in the distance of base pairs at the intercalation site [34]. Viscosity changes were measured using CT DNA with increasing concentrations of the complexes as shown in figure 5. The effects of ofloxacin, **1**, **2**, and **3** *versus* $[Ru(bpy)_3]^{2+}$ and EB (ethidium bromide) on the relative viscosity of rod-like DNA are shown in figure 5. $[Ru(bpy)_3]^{2+}$ interacts with DNA in a classical electrostatic effect with little changes in the viscosity of CT DNA and EB interacts with DNA in a classical intercalating mode showing increasing viscosity [35, 36]. The relative viscosity of the DNA does not change as the amounts of ofloxacin, **1**, **2**, and **3** increase, which is similar with $[Ru(bpy)_3]^{2+}$. This suggests that these complexes bind to DNA via electrostatic interaction [37].

4. Conclusion

Three Ru(II)–ofloxacin complexes were prepared in approximately 70% yield. According to the MTT results, the complexes exhibited acceptable inhibitory activity against HeLa cells, which were higher than that of ofloxacin. The CT DNA-binding properties of the complexes were studied using electronic spectra, fluorescence emission titrations, and viscosity experiments. Compared to the Cu(II)–ofloxacin complex $[Cu(ofloH)_2][(CuCl_2)_2$, it is found that the Ru complexes exhibit similar DNA-binding ability [38]. The results suggested that **1–3** interacted with CT DNA through the electrostatic effect. The ofloxacin complexes **1–3** exhibited antitumor activities with the direct ratio of DNA-binding properties, in which the increasing of substituent group or aromatic ring can enhance the antitumor

activity and DNA-binding affinity of Ru(II) complexes. Together, these results suggest that these types of complexes may exhibit an inhibitory effect on tumor cells through binding DNA.

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